Regulation of PP1 and Nucleic Acid binding by AKAP149 and PNUTS

Marie Rogne

Institute of Basic Medical Sciences
Department of Biochemistry
Faculty of Medicine
University of Oslo

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There is a phrase saying 'Never judge a book by the cover' and with those words in mind I will wish you all happy reading.

Oslo, November 2008

Marie Rogne

If you're not a part of the solution

Then you're a part of the precipitate

PAPERS INCLUDED

I **Landsverk, H.B., Kirkhus, M., Bollen, M., Küntziger, T and Collas, P. 2005**. *PNUTS enhances in vitro chromosome decondensation in a PP1-dependent manner*. **Biochem. J. 390, 709-717.**

II **Rogne, M., Landsverk, H.B., Van Eynde, A., Beullens, M., Bollen, M., Collas, P and Küntziger, T. 2006**. *The KH domain of A-kinase Anchoring Protein 149 Mediates RNA-dependent Self-association.* **Biochemistry. 45(50), 14980-14989.**

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IV **Rogne, M, Collas, P and Küntziger, T. 2009**. *QuickRIP – Cross-linked RNA immunoprecipitation*. **Manuscript.**

ABBREVIATIONS

APC	anaphase promoting complex
ATP	adenosine triphosphate
AKAP	A kinase anchoring protein
AMY1	associated of Myc-1
BAD	Bcl-2 antagonist of cell death
Blc-2	B-cell lymphoma 2
BSA	bovine serum albumin
C	catalytic subunit
cAMP	cyclic adenosine monophosphate
CD	chromosome decondensation
Cdc25	cell division cycle 25
CDK	cyclin-dependent kinase
cDNA	complementary deoxyribonucleic acid
CREB	cAMP response element-binding protein
D-	dual
DAPI	4'-6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EGF	epidermal growth factor
ER	endoplasmic reticulum
f-of	subunit of mitochondrial ATP synthase
GST	glutathione S-transferase
GFP	green fluorescent protein
HDAC	histone deacetylase
H1/2A/3/4	histone 1/2A/3/4
HKIF4A	human chromokinesin superfamily protein member 4A
hnRNP	heterogeneous ribonucleoprotein particles
KH	K homology
LPL	lipoprotein lipase
Mfn1/2	mitofusin 1/2
MnSOD	manganese superoxide dismutase
mStar	steroidogenic acute regulatory protein
mRNA	messenger ribonucleic acid
MYPT	myosine phosphatase targeting subunit
NE	nuclear envelope
Nek-2	NIMA related kinase 2
NIMA	never in mitosis gene A
Nipp1	nuclear inhibitor of PP1
OPA1	autosomal dominant optic atrophy
PDE	phosphodiesterase
PFK	phosphofructokinase
PKA	protein kinase A
PKC	protein kinase C
Plk1	polo like kinase 1
PML	promyelocytic leukemia
PNUTS	PP1 nuclear targeting subunit
PP1	protein phosphatase 1
PP2A	protein phosphatase 2 A
pRb	retinoblastoma protein
PP2B	protein phosphatase 2 B
PSF	polypyrimidine tract-binding protein associated splicing factor
PTB	polypyrimidine tract binding protein
PTPD1	protein tyrosine phosphatase D1
R	regulatory subunit
RanRp1	ran binding protein 1
RCC1	regulator of chromosome condensation 1
Repo-Man	recruits PP1 onto mitotic chromatin anaphase
RNA	ribonucleic acid
RNase	ribonuclease
RRM	RNA recognition motif
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SF2	splice factor 2
SMC	structural maintenance of chromosome
Tra2-beta1	transformer2-beta1
UTR	untranslated region

INTRODUCTION

The cell cycle encompasses all morphological and biochemical events that occur during the lifetime of a cell, including metabolism, growth and division. Progression through the cell cycle is regulated by internal clocks that control passage through multiple checkpoints. External stimuli also affect cell cycle progression. *In vivo*, stimulation from the cellular environment, mediated by hormones or neighboring cells, can promote or slow down cell division, and cause differentiation or cell death. Thus, cells need to establish a complex network of signaling pathways that register and process external cues in the context of their own internal clocks.

Transduction of an extracellular signal to intracellular effectors involves cascade of signaling events. This cascade includes the stimulation of surface receptor ligands on the target cell and transfer of the external signal to the cell interior. Intracellular second messengers propagate the signal to effector molecules, which in turn carry out the internal response to the external stimuli. Protein kinases and phosphatases are two major classes of effector proteins which alter the behavior of specific target proteins by phosphorylation and dephosphorylation, respectively. The phosphorylation status of many proteins contributes to define their localization, (substrate) binding properties or enzymatic activity. Protein phosphorylation and dephosphorylation are therefore involved in a wide range of cellular processes. Furthermore, as the level of protein phosphorylation can also act as a cellular signal, regulation and specificity of protein kinases and phosphatases become extremely important. Results presented in this thesis provide new biochemical and functional insights into two such phospho-regulating proteins involved in cell cycle progression, nucleic acid binding and mitochondrial distribution.

1. The cell cycle

Progression through the cell cycle

The eukaryotic cell cycle is typically divided into four phases, namely G_1, S, G_2 and M-phase. G_1 is the phase when cells prepare to synthesize a duplicate copy of DNA. Cells in G_1 can either exit the cell cycle G_0 and quiesce, or progress into S phase and replicate their DNA. Cells about to enter G_0 repress genes needed for entry into mitosis such as cyclins and cyclin-dependent kinases (Cdks); consequently, the cell leaves the cell cycle temporarily or permanently. Some cell types in mature organisms, such as parenchymal cells of the liver and kidney, enter G_0 semi-permanently and only start dividing again under specific circumstances (Jirtle *et al.*, 1981). Other cell types, such as epithelial cells, continue to divide throughout an organism's life (Stampfer *et al.*, 1993). M-phase is comprised of mitotic chromosome division and cytokinesis (**Fig. 1**). The purpose of mitosis is to enable successful and equal transmission of the genetic material into two daughter cells.

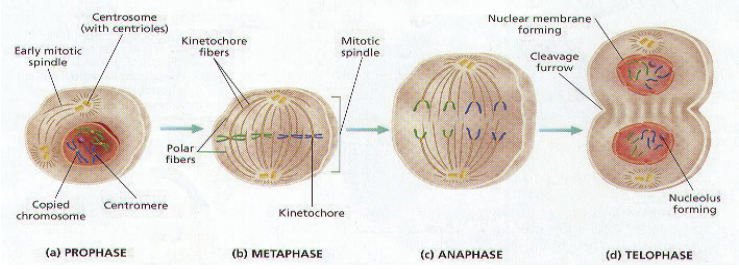

Figure 1. Stages of mitosis in a mammalian cell. In prophase **(a)** chromatin condenses into chromosomes, the nuclear envelope breaks down and the nucleolus disappears. In metaphase **(b)** chromosomes are aligned on the equatorial plane by the microtubuli attached to the centromeres. The two sister chromatids are attached to kinetochore microtubuli originating from the centrosomes at opposite ends of the cell. In anaphase **(c)** the two sister chromatids break apart and daughter chromosomes move towards opposite spindle poles. In telophase **(d)** the chromosomes decondense and the new nuclear envelope reforms (taken from www.sirinet.net/~jgjohnso/biologyI.html).

Regulation of Mitosis

Mitosis is primarily regulated by phosphorylation and proteolysis. The two are interrelated, as phosphorylation can regulate the proteolytic machinery, and mitotic kinases can be down-regulated by proteolysis (Peters, 2002).

Cdks are central components that coordinate cell-division. Mitotic cyclin-Cdk complexes drive events of early mitosis like chromosome condensation, nuclear envelope (NE) breakdown, and assembly of the mitotic spindle. Cdks complete their main functions by metaphase, when sister chromatid pairs are bi-oriented on the spindle, but held together by sister-chromatid cohesion (Sullivan and Morgan, 2007). In presumably all eukaryotes, sister chromatid separation and anaphase entry follow cyclin ubiquitin-dependent proteolysis to inactivate Cdk1 (Glotzer *et al.*, 1991). This event is mediated by the anaphase promoting complex (APC), a cell cycle-regulated ubiquitin ligase that assembles multi-ubiquitin chains on regulatory proteins such as securins and cyclins and thereby targets them for destruction (Glotzer *et al.*, 1991).

Entry into mitosis is characterized by a global increase in protein phosphorylation. Phosphorylation of several nuclear membranes, lamin and chromatin proteins correlates with dissociation of the NE from chromosomes (Courvalin *et al.*, 1992; Foisner and Gerace, 1993; Collas and Courvalin, 2000). Among DNA- and chromosome-associated proteins, histones H1, H2B, H3 and H4 are phosphorylated at mitosis entry (Dimitrov and Wolffe, 1996; Hsu *et al.*, 2000; Speliotes *et al.*, 2000; Murnion *et al.*, 2001; Giet and Glover, 2001; Barber *et al.*, 2004). The role of histone phosphorylation in chromosome condensation is unclear, but three models have been proposed.

One model hypothesizes that addition/removal of a phosphate group alters the charge of the nucleosome, which in turn affects intra- or inter-nucleosomal structure and stability. Such alterations could potentially facilitate chromatin condensation and/or sister chromatid separation (Prigent and Dimitrov, 2003). A second model hypothesizes that mitotic histone phosphorylation may directly contribute to the resolution of sister chromatids, possibly by enhancing flexibility of the chromatin fibers (Murnion *et al.*, 2001) or by increasing electrostatic repulsion between the two chromatids (Swedlow and Hirano, 2003). This in turn contributes to chromatin condensation and sister chromatid separation (Swedlow *et al.*, 2003). In the third model, histones serve as docking sites for effector proteins that mediate chromatin condensation and separation (Kanno *et al.*, 2004). This illustrates the importance of histone modifications in signal transduction cascades and in mediating protein binding to chromatin (Fischle *et al.*, 2003; Kanno *et al.*, 2004).

NE breakdown and lamin phosphorylation resulting in lamin depolymerization is another important regulatory event in mitosis. In vertebrates, NE disassembly defines the transition between prophase and prometaphase (Foisner, 2003). Lamin depolymerization is important for the dissociation of proteins from chromatin and normal NE breakdown. Accordingly, cells expressing lamin mutants that can not be phosphorylated by mitotic kinases do not efficiently disassemble the NE (Foisner, 2003).

Numerous other proteins are known to regulate entry into mitosis, and several have been shown to be important or necessary for proper cell progression through mitosis. For instance, Aurora B contributes to chromosome condensation by phosphorylating H3 and by regulating the condensin complex (See page 14) (Takemoto

et al., 2007). In *Schizosaccharomyces pombe (S. pombe)*, *Caenorhabditis elegans (C. elegans)* and *Drosophila melanogaster (D.melanogaster)*, condensins fail to associate with chromatin in metaphase if phosphorylation by Aurora B is inhibited (Giet *et al.*, 2001; Petersen *et al.*, 2001; Kaitna *et al.*, 2002). Furthermore, phosphorylations of the Never in Mitosis Gene A (NIMA) family of proteins by Cdk1 and of polo kinases family (e.g., polo-like kinase 1 or plk1) by an unidentified kinase, are essential for mitosis entry (Ferrari, 2006).

Conversely, protein dephosphorylation is required for chromatin decondensation and reentry into interphase. This includes H3 dephosphorylation by protein phosphatase 1 (PP1) (Murnion *et al.*, 2001). An emerging concept is that precise ordering of late mitosis events depends, at least in part, on the order in which Cdk substrates are dephosphorylated and APC targets are destroyed (Sullivan *et al.*, 2007). Thus, the reversible phosphorylation of chromatin-associated proteins through mitosis is widespread, essential and complex.

Mitotic chromosome condensation

Upon entry into mitosis, chromosomes undergo reversible condensation. Human mitotic chromosomes are compacted 15 000-20 000-fold, as defined by the ratio of DNA to chromosome length (Li *et al.*, 1998). This massive compaction is essential to ensure correct transmission of genetic information during cell division. A component essential for proper progression through mitosis is topoisomerase II (Uemura *et al.*, 1987;Adachi *et al.*, 1991; Saka *et al.*, 1994; Saitoh *et al.*, 1995). Topoisomerase II is postulated to be involved in DNA supercoiling, individualization of chromosomes (Gimenez-Abian *et al.*, 1995; Losada *et al.*, 2002) and separation of sister chromatids in anaphase (Uemura and Yanagida, 1984; Holm *et al.*, 1985; Shamu and Murray, 1992). A family of adenosine triphosphatases (ATPases), called structural maintenance of chromosome (SMC) proteins, is also essential for normal progression through mitosis (Hirano, 1999; Hirano, 2002). SMC protein complexes are conserved from yeast to mammals and were first identified in *Saccharomyces cerevisiae (S. cerevisiae)* (Strunnikov *et al.*, 1993; Strunnikov *et al.*, 1995). There are two known SMC protein complexes that each consist of two SMC and three non-SMC subunits (**Fig. 2**). SMCs are involved in sister-chromatid cohesion, chromosome assembly, DNA recombination and repair and gene dosage compensation (Hagstrom and Meyer, 2003)

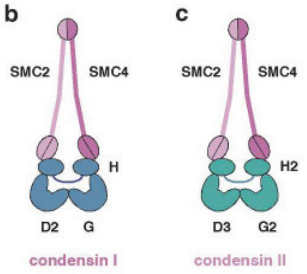

Figure 2. Composition of condensin I and II remodeling complexes. Condensin I and II contain the same SMC subunits but different non-SMC subunits (Losada and Hirano, 2005).

The exact structure and formation of mitotic chromosomes is not known, but at least two hypotheses exist. One hypothesis proposes DNA compaction around a protein backbone and has three main models (**Fig. 3**): 1) the helical folding model suggests helical coiling of the 30 nm chromatin fiber into progressively larger structures (Sedat and Manuelidis, 1978); 2) the radial loop model proposes that DNA is connected in loop domains to a protein scaffold (Paulson and Laemmli, 1977; Marsden and Laemmli, 1979); 3) the combined radial loop-helical folding model suggests a combination of the two previous models (Rattner and Lin, 1985; Boy de la and Laemmli, 1988).

The second hypothesis monitors changes in chromosome elasticity and deformability induced by nuclease digestion and physical stretching of individual chromosomes (Houchmandzadeh *et al.*, 1997; Poirier *et al.*, 2002a; Poirier and Marko, 2002a; Poirier and Marko, 2002b; Poirier *et al.*, 2002c). From force measurements and chromosome micromanipulation experiments, shape, structure and mechanical properties of chromosomes were shown to depend on the structural integrity of DNA, ruling out a primary structural role for a physically contiguous protein network embedded within the chromosome (Poirier *et al.*, 2002b). However, these results do not rule out the possibility of a dynamic protein assembly that could drive chromatin folding into chromosomes of a defined shape (Jessberger, 2003).

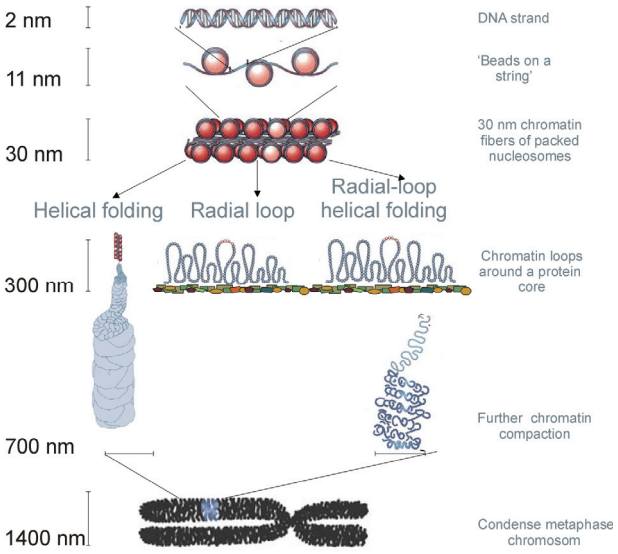

Figure 3. Three models for chromosome structure and formation. The helical folding model suggests helical coiling of the 30 nm fiber into progressively larger structures (Sedat *et al.*, 1978). The radial loop model proposes that DNA is connected in loop domains to a protein scaffold (Paulson *et al.*, 1977;Marsden *et al.*, 1979). The combined radial loop-helical folding model suggests a combination of the two previous models (Rattner *et al.*, 1985;Boy de la *et al.*, 1988). Modified from home.planet.nl/~gkorthof/korthof59.htm.

Most text book models of mitotic chromosome structure feature the radial loop model of mitotic chromosome condensation (**Fig. 3**) (Paulson *et al.*, 1977). There are major questions regarding this model however, since the major identified components of the protein scaffold are topoisomerase II and SMC2. Neither topoisomerase II nor SMC2 are obvious candidates as building blocks for a structural scaffold network as they do not form an axial chromosome staining pattern until late prophase, when chromosome compaction is nearly complete. Studies in mammals, chicken, *C. elegans* and *D. melanogaster* have led to the conclusion that compaction of chromatin and formation of a longitudinal chromosome axis proceeds in the absence of topoisomerase II or SMC2, albeit with serious sister chromatid separation and chromosome segregation defects

(Steffensen *et al.*, 2001; Hagstrom *et al.*, 2002; Coelho *et al.*, 2003; Hudson *et al.*, 2003; Hirota *et al.*, 2004; Gassmann *et al.*, 2004; Savvidou *et al.*, 2005).

The radial loop model has recently been strengthened by the discovery of potential components of the mitotic chromosome scaffold. Based on salt and detergent extraction of chromatin, a list of 79 components of the chromosome scaffold fraction have been published (Gassmann *et al.*, 2005), of which 30 have not previously been identified as components of mitotic chromosomes, and 15 are uncharacterized. Interestingly, two of the proteins identified, nuclear protein (P30) and human kinesin superfamily protein member 4A (HKIF4A) have previously been shown to be involved in proper chromosome condensation (Mazumdar *et al.*, 2004; Gassmann *et al.*, 2005). Furthermore, overexpression of P30 or HKIF4A knockdown leads to hypercondensed chromatin (Mazumdar *et al.*, 2004; Gassmann *et al.*, 2005). The incomplete picture concerning both chromatin condensation and maintenance of mitotic chromosomes imply the existence of another, as yet undiscovered molecular mechanism driving chromosome condensation.

In vitro systems have been used to study chromosome condensation and decondensation. *Xenopus laevis (X. laevis)* pronulei or somatic nuclei incubated in a mitotic Ca^{2+}-free *X. laevis* egg extract undergo NE breakdown, chromosome condensation and spindle formation in a calcium-dependent manner (Lohka and Masui, 1984a; Lohka and Maller, 1985). On the contrary, chromosome decondensation can be observed after incubation of *X. laevis* sperm nuclei in calcium-activated extract (Lohka *et al.*, 1984a). Further, it has been shown by us and others that *in vitro* chromosome

condensation and decondensation can take place in extracts from Hela cells (Wood and Earnshaw, 1990; Collas *et al.*, 1999a; Steen *et al.*, 2000a).

These observations indicate that chromosome condensation and decondensation can be simulated *in vitro* using exogenous substrates in lysates from eggs or somatic cells. Work presented in this thesis (paper I) relies on an extract derived from interphase HeLa cells to indicate the role of a nuclear protein phosphatase regulator on decondensation of mitotic chromosomes.

Chromosome decondensation

Two major events of mitosis exit are chromosome decondensation and NE reassembly. Molecular events involved in mitotic chromosome decondensation remain largely unresolved, but as expected, many chromosome-related events that take place at mitosis entry are reversed at mitosis exit. Ran binding protein 1 (RanBP1) and regulator of chromosome condensation 1 (RCC1) are two components of the small GTPase Ran cycle which controls nuclear protein import and export, but which is also important in mitosis for spindle assembly and chromosome decondensation (**Fig. 4**) (Zhang *et al.*, 2002; Quimby and Dasso, 2003; Clarke and Zhang, 2008). Ran mutants with deficient GTPase domain, or RCC1 mis-localization from chromosomes produces defects in chromosome alignment at metaphase or multipolar spindles (Zhang *et al.*, 1999; Wilde and Zheng, 1999; Ohba *et al.*, 1999; Carazo-Salas *et al.*, 1999). Moreover RCC1 depletion from *X. laevis* extracts or RanBP1 overexpression in mammalian cells leads to chromosome decondensation failure (Sazer and Nurse, 1994; Battistoni *et al.*, 1997; Zhang *et al.*, 2002). Furthermore, direct binding of RCC1 to chromatin, followed by local generation

of Ran-GTP and GTP hydrolysis by Ran, induces chromatin decondensation, membrane vesicle recruitment to chromosomes, reformation of the NE and reassembly of nuclear pore complexes (Hetzer *et al.*, 2000; Zhang and Clarke, 2000). Collectively, these observations imply an important role for RCC1 and Ran in the process of chromosome decondensation.

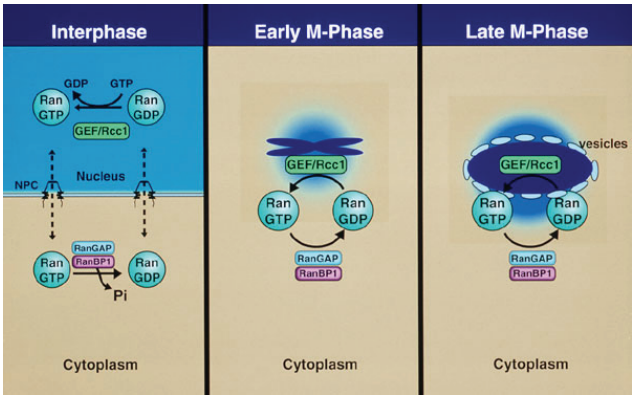

Figure 4. RanGTP throughout the cell cycle (Taken from webpage of Mattaj Group, www-db.embl.de/jss/EmblGroupsHD/g_45.html). During interphase Ran is located on both sides of the NE and actively drives transport through the nuclear pore complex. When the NE breaks down as the cell enters mitosis RanGTP is important for mitotic spindle assembly. Further as mitosis reaches anaphase RanGTP is essential to reform the nuclear pore complexes and the NE.

Ubiquitination and degradation of mitotic cyclins at metaphase that inactivate Cdks, are another prerequisite for proper chromosome decondensation (Sullivan *et al.*, 2007). This allows dephosphorylation of the many Cdk substrates necessary for normal chromosome and spindle movements in anaphase, and for spindle disassembly, reformation of NE and chromosome decondensation through telophase (Sullivan *et al.*, 2007). Cyclin oscillations through the cell cycle represent a good example of the essential

spatiotemporal regulation of protein phosphorylation and dephosphorylation to ensure proper cell division, differentiation and viability.

2. Protein phosphorylation and dephosphorylation

Intracellular signaling through cAMP-dependent protein kinase A

Approximately one third of eukaryotic proteins are regulated by phosphorylation. Cyclic AMP (cAMP)-dependent protein kinase, or protein kinase A (PKA), is a central and well studied Serine/Threonine (Ser/Thr) protein kinase (Skalhegg and Tasken, 1997; Kim *et al.*, 2006). The intracellular messenger cAMP regulates numerous biological processes including motility, metabolism, differentiation, immune function, insulin secretion and cardiac contraction (Meinkoth *et al.*, 1993; Montminy, 1997; Diviani, 2008; Lafontan *et al.*, 2008). Production of cAMP is primarily triggered by agonist-induced activation of transmembrane G protein-coupled receptors followed by activation of adenylyl cyclases at the inner side of the plasma membrane. cAMP activates a limited number of effectors including PKA which, by phosphorylating a multiplicity of nuclear and cytoplasmic targets, mediates numerous biological effects (Traish *et al.*, 1997; Zaccolo *et al.*, 2006; Chiaradonna *et al.*, 2008).

The PKA holoenzyme is composed of two regulatory and two catalytic subunits. The different isoforms of the regulatory (RIα, RIβ, RIIα, RIIβ) and catalytic (Cα, Cβ, Cγ) subunits possess distinct physical and biological properties. They are differentially expressed and their combination creates different isoforms of PKA holoenzymes (Tasken and Aandahl, 2004; Kim *et al.*, 2006). PKA type I holoenzyme consists of RI subunits and PKA type II holoenzymes contain RII subunits. Specificity of PKA is largely

determined by the structure and properties of the regulatory subunits, whereas the catalytic subunits exhibit similar kinetic features and substrate specificities (Kim *et al.*, 2006). Both PKA types I and II holoenzymes are activated by cAMP binding to the regulatory (R) subunit dimer. This releases the activated catalytic subunit to enable substrate phosphorylation (Vermeulen *et al.*, 2003; Wong and Scott, 2004; Tasken *et al.*, 2004; Zaccolo *et al.*, 2006).

Phosphodiesterases (PDEs) are the only known cyclic nucleotide-degrading enzymes and, therefore, are critical regulators of cAMP intracellular homeostasis by hydrolyzing cAMP to AMP (Houslay and Adams, 2003; Conti and Beavo, 2007). It has been reported that cells contains a large number of conserved PDEs, each of them with several isoforms, able to hydrolyze cAMP (Soderling and Beavo, 2000). Furthermore, isoform-specific knockdown in the PDE4 subfamily shows distinct phenotypes, indicating that the large PDE isoform diversity and numbers may control the cAMP pool in a spatio-temporal manner (Jin and Conti, 2002; Zaccolo and Movsesian, 2007).

AKAP's: multiscaffoling proteins that anchor PKA

Given the large diversity of PKA targets, precise regulation and confined activity in PKA signaling is essential for specificity of the intracellular response. A major family of proteins regulating PKA signaling consists of A kinase-anchoring proteins (AKAPs).

AKAPs are a structurally diverse family of functionally related proteins with more than 50 members including splice-variants. AKAPs are defined on the basis of their ability to bind radioactively labeled PKA regulatory subunits in overlay assays, and co-precipitate PKA catalytic activity (Hausken and Scott, 1996; Wong *et al.*, 2004; McConnachie *et al.*, 2006). Their functional significance also involves targeting of the PKA holoenzyme to specific subcellular compartments to focus PKA activity towards relevant substrates. This, therefore, provides spatial and temporal regulation of PKA signaling (Colledge and Scott, 1999; Tasken *et al.*, 2004; Langeberg and Scott, 2005). Anchored pools of PKA are selectively activated by pools of cAMP that are spatially regulated by the activity of PDEs (Tasken *et al.*, 2004; Zaccolo *et al.*, 2007).

AKAPs share at least three common features. 1) A conserved PKA binding motif that consists of a 14-20 amino-acids amphipatic helix able to interact with the amino-terminus of the regulatory subunit dimer (Carr *et al.*, 1992; Newlon *et al.*, 1999; Newlon *et al.*, 2001; Fayos *et al.*, 2003; Taylor *et al.*, 2005). 2) A unique targeting domain directing the AKAP-PKA complex to discrete subcellular sites including the plasma-membrane, mitochondria, centrosomes, Golgi, endoplasmic reticulum (ER) and the nucleus (**Fig. 5**) (Feliciello *et al.*, 2001; Wong *et al.*, 2004). 3) Most AKAPs have the ability to form multivalent signal transduction complexes by interacting with additional binding partners and signaling molecules such as protein kinase C (PKC), PDEs, small

GTPases, PP1, protein phosphatase 2A (PP2A) or protein phosphatase 2B (PP2B) (Colledge *et al.*, 1999; Diviani and Scott, 2001; Wong *et al.*, 2004; Tasken *et al.*, 2004; Diviani, 2008). Hence, the AKAP complexes have emerged as multi-scaffolding adaptor molecules with an essential role in the spatial and temporal integration of effectors and substrates. AKAPs provide a high level of specificity and sequential regulation to the cAMP/PKA signaling pathway (Tasken *et al.*, 2004).

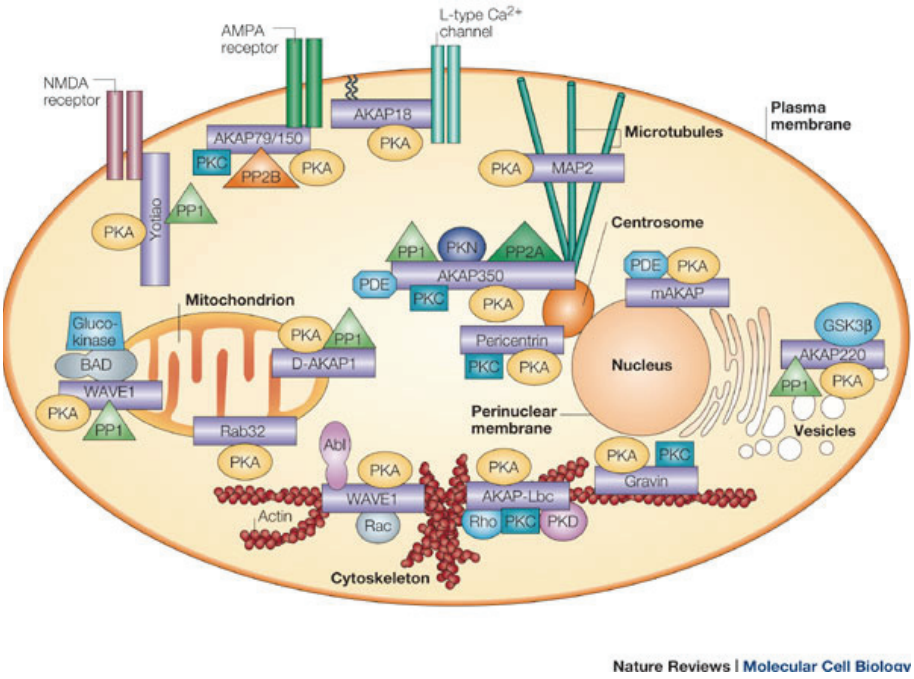

Figure 5. Subcellular localization and binding partners for some AKAP scaffolding complexes (Taken from, Wong and Scott, 2004).

Protein phosphatases

The action of protein kinases is counterbalanced by dephosphorylation mediated by protein phosphatases. The nucleus contains many phosphatases involved in cell cycle progression, DNA replication, transcription and RNA processing. Surprisingly, there are 2-5 times fewer protein phosphatases than kinases, and when analysis is limited to Ser/Thr phosphatases, there are ~20 times fewer Ser/Thr phosphatases than kinases (Bollen, 2001). However, the catalytic subunits of protein phosphatases can form complexes with over 50 different regulatory subunits (also referred to as 'R') in an exclusive or combinatorial manner (Bollen, 2001; Cohen, 2002b). This enables a very accurate spatiotemporal regulation of dephosphorylation events in the cell.

Protein phosphatase 1

PP1 is one of the most conserved enzymes in eukaryotes (Ceulemans and Bollen, 2004) **(Fig. 6)**. PP1 is a Ser/Thr phosphatase involved in the regulation of cellular processes such as muscle contraction, gene expression, glycogen metabolism, mRNA splicing and neurotransmission (Ceulemans *et al.*, 2004). PP1 is also implicated in RNA/pre-mRNA processing (Hirano *et al.*, 1996; Boudrez *et al.*, 2000; Novoyatleva *et al.*, 2008), mitosis exit (Fernandez *et al.*, 1992) and NE assembly (Steen *et al.*, 2000b). Mammals have three PP1 genes, encoding the PP1α, PP1β/δ and PP1$\gamma_{(1 \text{ and } 2)}$ isoforms. With the exception of the testis-enriched PP1γ_2, the mammalian isoforms are ubiquitously expressed into multiple cellular compartments. Even though isoforms of the catalytic subunit (PP1c, referred to as PP1) display ~90% sequence identity at the amino acid level, the isoforms probably execute distinct functions *in vivo* as some regulatory subunits bind PP1 in an

isoform-specific manner and the phenotype of a functional loss of PP1 is isoform-specific (Schillace and Scott, 1999; Cheng *et al.*, 2000; Raghavan *et al.*, 2000).

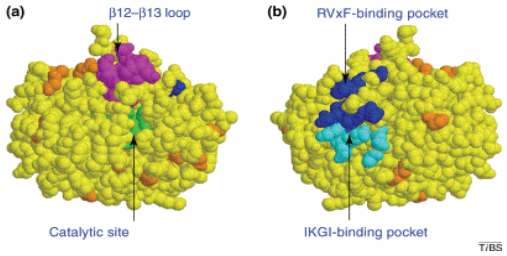

Figure. 6. Structure of PP1c. Two views of residues 7-300 of the α isoform of mammalian PP1c are shown. **(a)** The catalytic site (green) and the β12-β13 loop (magenta), required for inhibition by various inhibitory R subunits are shown. **(b)** The RVXF binding pocket (purple) and the IKGI sequence in the N-terminal domain of Inhibitor-2 (cyan) are shown (Bollen, 2001). Other surface residues (orange) are essential for optimal interaction with R subunits (Ramaswamy et al., 1998) (taken from Bollen 2001).

The catalytic subunits of PP1 do not exist freely in the cell, but associate with many R subunits to form distinct multimeric holoenzymes. The PP1 holoenzyme consists of a single catalytic subunit and one or two R subunits (Aggen *et al.*, 2000; Bollen, 2001; Ceulemans *et al.*, 2002; Cohen, 2002a). R subunits can act as inhibitors of PP1c, activators of PP1c or act as substrate specifiers that increase PP1 activity towards certain substrates and decrease it towards others (Ceulemans *et al.*, 2004).

Various examples include: nuclear inhibitor of PP1 (NIPP1) is a nuclear targeting regulatory subunit implicated in RNA processing (Bollen and Beullens, 2002). Myosine phosphatase targeting (MYPT) subunit (Hirano *et al.*, 1999) and AKAP149 (Steen *et al.*, 2003) act as substrate specifiers by promoting PP1 activity towards myosin light chain and B-type lamins, respectively, whereas both inhibit PP1 activity towards phosphorylase *a in vitro* (Johnson *et al.*, 1997; Steen *et al.*, 2003). NIMA related kinase 2 (Nek2), involved in maintenance and modulation of centrosome architecture (Mi *et al.*, 2007), is a

PP1 regulatory subunit in addition to being a PP1 substrate (Helps *et al.*, 2000). For other PP1 interactors, such as phosphofructokinase (PFK), the retinoblastoma protein (Rb), and Sla1, it is not yet clear whether they are regulators and/or substrates of PP1, or whether they bind directly to PP1 or via another interactor (Ceulemans *et al.*, 2004). The large diversity of PP1 holoenzymes provides the cell with a precise regulation of PP1-mediated dephosphorylation in a localization- and substrate-specific manner.

PP1 regulatory subunits

An interesting question is how PP1 can interact with a large selection of subunits that are not structurally related and that have distinct effects on the activity and substrate specificity of the phosphatase. Evidence has demonstrated that 1) R subunits typically bind to PP1c via a short (4-6 residues) degenerate sequence motif 2) most R subunits have multiple interaction sites with PP1; and 3) R subunits can share PP1 interaction sites. The best characterized PP1 binding site within R subunits, is the so-called 'RVXF' motif (where X is any amino-acid), which forms a hydrophobic groove. The RVXF motif serves as an initial anchor for PP1 binding and thereby promotes, sometimes cooperatively, binding to secondary sites, which are often of lower affinity for PP1 (Bollen, 2001; Wakula *et al.*, 2003).

PP1 is involved in RNA regulation and is essential for mRNA splicing

PP1 activity has been shown to be essential for RNA splicing (Mermoud *et al.*, 1992; Boudrez *et al.*, 2000; Novoyatleva *et al.*, 2008) and in the nucleus, PP1 interacts with the splicing factor polypyrimidine tract-binding protein-associated splicing factor (PSF)

(Hirano *et al.*, 1996). PP1-mediated dephosphorylation is involved in structural rearrangements necessary for the transition from the first to second step of splicing. PP1 associates with complexes formed on pre-mRNA (Hirano *et al.*, 1996). However, binding partners and dephosphorylation targets of the phosphatase in the spliceosome are largely unknown.

Transformer2-beta1 (tra2-beta1), similar to an SR-protein (family of splice-factors essential in splicing that always contains a SR domain) that regulate splice site selection by recruiting regulatory proteins to exon sequences, contains a conserved PP1 binding motif (Novoyatleva *et al.*, 2008). Interestingly, this binding RVDF motif is located in the RNA recognition motif (RRM) and binds PP1 (Novoyatleva *et al.*, 2008).The PP1-binding motif is also conserved in the beta-4 strand of eight other RRMs, including those of SF2/ASF, SRp30c and polypyrimidine tract-binding protein (PTB) (Novoyatleva *et al.*, 2008).This illustrates an important role for PP1 regulation in RNA metabolism.

Involvement of PP1 in chromosome dynamics during the cell cycle

PP1 is important for mitosis exit in yeast, fungi and mammalian cells (Ohkura *et al.*, 1989; Doonan and Morris, 1989; Fernandez *et al.*, 1992; Alberts *et al.*, 1993; Hisamoto *et al.*, 1994; Steen *et al.*, 2000b), and a significant part of PP1 co-localizes with chromosomes, centromeres and spindle at mitosis (Fernandez *et al.*, 1992; Trinkle-Mulcahy *et al.*, 2001). PP1 has been shown to dephosphorylate Rb allowing cells to exit mitosis, (Rubin *et al.*, 1998; Berndt, 1999;Rubin *et al.*, 2001; Udho *et al.*, 2002). PP1 also maintains nuclear lamins in a dephosphorylated form at NE throughout G1, thereby

maintaining nuclear integrity (Steen *et al.*, 2003). Furthermore, inhibition of PP1 activity at metaphase results in metaphase arrest (Fernandez *et al.*, 1992), whereas PP1 inhibition during anaphase prevents completion of cell division. Conversely, increasing level of PP1 at anaphase accelerates completion of cytokinesis (Fernandez *et al.*, 1992). The 'recruits PP1 onto mitotic chromatin at anaphase' (Repo-Man)-PP1 complex was also recently shown to play a critical role in the maintenance of chromosome architecture during mitosis, probably at the level of condensin regulation (Vagnarelli *et al.*, 2006; Trinkle-Mulcahy *et al.*, 2006). In Paper I in this thesis, PP1 is proposed to regulate chromatin decondensation at the end of mitosis through the R subunit PNUTS/p99.

PP1 nuclear targeting subunit (PNUTS)

PNUTS/R111/p99 (Kreivi *et al.*, 1997; Allen *et al.*, 1998; Kim *et al.*, 2003) is a nuclear PP1 targeting and R subunit found in most tissues in human and rat (Allen *et al.*, 1998). Yeast two-hybrid screening and immunoprecipitation assays (Allen *et al.*, 1998) show that both PP1α and PP1γ isoforms interact with PNUTS (Allen *et al.*, 1998). Expression profile of PNUTS in rat brain cortex development shows a continual decrease in expression to a low protein level in the adult, which suggests a role for PNUTS in brain development (Allen *et al.*, 1998). Interestingly, PNUTS has been found to be down-regulated in brain samples from patients with Alzheimer disease as well (Raha-Chowdhury *et al.*, 2005). PNUTS has also been implicated in the regulation of p53 expression and phosphorylation under mild hypoxia conditions, a function dependent on an intact PP1 binding domain (Lee *et al.*, 2007b). Furthermore PNUTS:PP1 dissociation under mild hypoxia leads to activation of PP1, dephosphorylation of Rb, and cell cycle

arrest in G1 (Lee *et al.*, 2007b). PNUTS, therefore, may represent an interesting new target for tumor treatment by enhancing sensitivity to hypoxia-induced cell death (Lee *et al.*, 2007b).

PNUTS also acts as a substrate specifier of PP1. For instance, PNUTS suppresses the phosphatase activity of PP1 toward phosphorylase *a* by >90% (Kreivi *et al.*, 1997), indicating that PNUTS is a PP1 inhibitor towards this substrate *in vitro*. On the other hand, Paper I in this thesis suggests that PNUTS associated with PP1 does not inhibit but rather activates PP1 in the process of chromosome decondensation, at least in a cell-free system. The PP1 interaction domains of PNUTS consists of a degenerate RVXF (LTVTW) motif and an inhibitory domain (ETARRL), mapped to, respectively, amino acids 396-401 (Kreivi *et al.*, 1997; Kim *et al.*, 2003) and 445-450 (Kim *et al.*, 2003). Association of PNUTS with the PP1 catalytic subunit is regulated by phosphorylation through the PKA consensus motif containing T398, where by phosphorylation of T398 by PKA reduces PP1c-PNUTS association (Kim *et al.*, 2003).

Subcellular localization of PNUTS varies during the cell cycle. During interphase, PNUTS is intranuclear (Kreivi *et al.*, 1997; Allen *et al.*, 1998). Upon entry into mitosis, PNUTS is dispersed in the cytoplasm and is clearly dissociated from chromosomes (Kreivi *et al.*, 1997; Allen *et al.*, 1998). PNUTS immunolabeling appears diffuse at anaphase, whereas at telophase PNUTS relocalizes to the reforming nucleus (Allen *et al.*, 1998). Paper I shows the cell cycle-dependent localization of PNUTS and suggests a role for PNUTS on chromosome dynamics in a cell-free nuclear reassembly system. These findings form a working hypothesis for Paper I presented in this thesis.

A kinase anchoring protein 149 (AKAP149)/AKAP121/dAKAP1

Description

AKAP149 is a dual PKA-anchoring and PP1-binding protein, and in addition functions as a substrate-specifier. AKAP149 contains 903 amino acids and harbor several functional domains. AKAP149 has an N-terminal (residues 1-30) mitochondrial/NE/ER anchoring domain, two consensus PP1-binding motifs (residues 153-157 and 627-631), a RII/RI binding domain (residues 355-376), a leucine zipper (residues 310-338) and single KH (residues 612-659) and TUDOR (residues 708-829) motifs in the far C-terminal (**Fig. 7**)(Trendelenburg *et al.*, 1996).

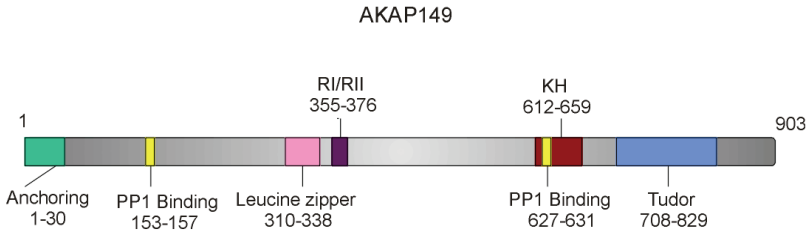

Figure 7. AKAP149 with interaction domains and functional domains identified to date.

S-AKAP84 (Lin *et al.*, 1995), D-AKAP1 (Huang *et al.*, 1999), AKAP149 (Trendelenburg *et al.*, 1996) and its mouse homolog AKAP121 (Chen *et al.*, 1997) arose by alternative splicing of the *akap1* gene (**Fig. 8**.). These isoforms share a 525 amino acid NH_2-terminal core, containing the PKA binding domain, but differ in their COOH- and NH_2-terminal sequences. At least four different COOH-terminal splice-variants have been identified, in addition to several NH_2-terminal splice-variants (Huang *et al.*, 1999; Ma and Taylor, 2008). S-AKAP84, D-AKAP1, AKAP121 and AKAP149 all target mitochondria (Cardone *et al.*, 2002; Cardone *et al.*, 2004; Rogne *et al.*, 2006; Dyson *et*

al., 2008). However, several reports in mouse indicate that two alternative splice variants in the NH_2-terminus of D-AKAP1 direct it either to mitochondria (AKAP1c) or to the endoplasmic reticulum (ER) (AKAP1d) (Huang *et al.*, 1999; Ma and Taylor, 2002; Ma *et al.*, 2008). Moreover, a PKA-RII overlay assay has identified AKAP149 in purified NE extracts from HeLa cells (Steen *et al.*, 2000b). This suggests that AKAP149 together with its splice variants (**Fig. 8**) localizes to the mitochondria or ER/NE network presumably depending on its NH_2-terminal localization signal.

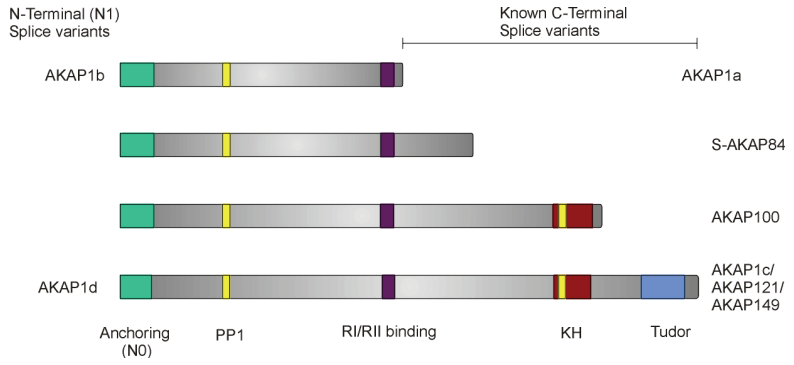

Figure 8. Characterized splice variants of D-AKAP1. AKAP1 has 4 different C-terminal splice variants, two of which (AKAP1a and AKAP1c/AKAP121/AKAP149) also contain a NH_2-terminal splice variant (AKAP1b and AKAP1c, respectively). AKAP1c and AKAP121/149 are the same protein, although they derive from slightly different mRNAs.

S-AKAP84 is testis specific (Furusawa *et al.*, 2002), and AKAP1d has only been detected in liver (Huang *et al.*, 1999). On the other hand AKAP1c/AKAP149/AKAP121 expression is more ubiquitous and has been detected in heart, brain, lung, liver, skeletal muscle, kidney, testis, but not in spleen (Huang *et al.*, 1999). AKAP149/121 is also expressed in oocytes under maturation, in brown fat tissue and differentiated adipocytes (Ranganathan *et al.*, 2002; Yukitake *et al.*, 2002; Chaudhry *et al.*, 2002; Webb *et al.*,

2008). Thus, when considering all isoforms, the distribution of AKAP149 is relatively ubiquitous.

Binding partners of AKAP149

AKAP149 emerges as a multiscaffolding AKAP, with a growing number of known interaction partners (Lin *et al.*, 1995; Trendelenburg *et al.*, 1996). AKAP149 binds both the RI and RII regulatory subunits of PKA *in vitro,* which makes it a dual AKAP (Huang *et al.*, 1999; Carlson *et al.*, 2003). AKAP149 contains two PP1 binding motifs, the NH_2-terminal one associates with PP1 in an *in vitro* overlay assay. PP1 binding was also shown by immunoprecipitation in HeLa cells (Steen *et al.*, 2000b; Steen *et al.*, 2003; Kuntziger *et al.*, 2006). AKAP149 was shown to be a substrate specifier of PP1, since the AKAP149:PP1 complex inhibits PP1 activity towards phosphorylase *a* but enhances PP1 activity towards B-type lamins (Steen *et al.*, 2003).

AKAP149 co-precipitates PKC, and *in vitro* assays indicate that AKAP149-bound PKA and PKC serine phosphorylate immunoprecipitated AKAP149, and that PKC phosphorylation promotes dissociation of PP1 from the AKAP (Kuntziger *et al.*, 2006). These results suggest a putative temporally and spatially controlled mechanism where PKC promotes the release of PP1 from the NH_2-terminal of AKAP149 presumably in a cell-cycle-dependent manner (Kuntziger *et al.*, 2006).

Moreover, AKAP121/149 targets the Src Tyr kinase to mitochondria via association with protein tyrosine phosphatase D1 (PTPD1) (Cardone *et al.*, 2004). PTPD1 is a widely expressed cytostatic non-receptor Tyr phosphatase, a positive regulator of Src

signalling and a key component of the epidermal growth factor (EGF) transduction pathway (Cardone *et al.*, 2004). By binding and targeting the phosphatase to mitochondria, AKAP121 may be involved in the regulation of the Src-dependent EGF transduction pathway (Livigni *et al.*, 2006). In one report, AKAP121/149 was also shown to bind PDE4A in T lymphocyte cells (Asirvatham *et al.*, 2004). The biological function for this interaction is unknown at present but provides interesting new possibilities for a fine-tuned regulation of PKA-mediated phosphorylation in mitochondria.

Other binding partners for AKAP149 in addition to the ones described above are known. The NH_2-terminal mitochondrial targeting domain of AKAP84/149 associates with α-tubulin (Cardone *et al.*, 2002) and the RII binding domain binds with associated of Myc-1 (AMY1) (Furusawa *et al.*, 2001; Furusawa *et al.*, 2002). Association with AMY1 and PKA to AKAP149 is mutually exclusive, causing bound AMY-1 to inhibit AKAP149-related PKA activity in mitochondria (Furusawa *et al.*, 2002). As discussed below the KH domain of AKAP149 also binds RNA. Taken together, the growing number of AKAP149 interaction partners leads to the postulation that AKAP149 functions as a scaffolding protein for several signalling molecules (**Fig. 9**)

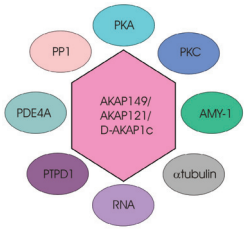

Figure 9. AKAP 149 and known binding partners.

Biological functions

A growing number of reports implicate AKAP149 in different biological functions. Functions involving the PKA binding domain, PP1 binding motif and the K Homology (KH) domain are the most described. AKAP149 directed PKA localization in the outer mitochondrial membrane increases PKA-dependent phosphorylation and inactivation of the proapoptotic protein Blc-2 agonist in the cell (BAD) and enhances cell survival (Harada *et al.*, 1999; Affaitati *et al.*, 2003; Livigni *et al.*, 2006). Furthermore, regulation of AKAP121/149 protein levels by ubiquitination and proteosomal degradation is important to allow PKA activity to fluctuate rapidly in the cell in response to changes in oxygen levels (Carlucci *et al.*, 2008). A role for AKAP121/149 in oxidative metabolism and cell survival has also recently been proposed (Carlucci *et al.*, 2008). Interestingly AKAP149 is a substrate of active caspases-3, -8 -and -10 *in vitro* and *in vivo* when apoptosis is induced (Yoo *et al.*, 2008). Caspases play a central role by transducing and amplifying the intracellular death signal, and apoptosis is executed as a consequence of caspase-mediated cleavage of multiple cellular substrates (Yoo *et al.*, 2008). Collectively, the results reveal the importance of AKAP149 in cell survival. In addition, AKAP1 knock-out mice with an interrupted PKA localization to mitochondria show oocyte maturation failure, supposedly due to the inability to remove PKA from the nucleus in meiosis division two (Newhall *et al.*, 2006; Webb *et al.*, 2008). Consequently females are sterile (Newhall *et al.*, 2006). These results again demonstrate the importance of spatiotemporal regulation of PKA in many important biological functions.

As mentioned earlier, previous work from our laboratory has shown that AKAP149 acts as a PP1 targeting subunit to the reforming NE *in vitro* at mitosis exit (Steen *et al.*, 2000b; Steen *et al.*, 2003), and that NE reassembly at the end of mitosis requires targeting of B-type lamin and PP1 to the NE by AKAP149 (Steen *et al.*, 2000b). Furthermore, PP1 remains associated with NE bound AKAP149 throughout G1 but is released from AKAP149 during S phase entry, as AKAP149 becomes Ser-phosphorylated (Steen *et al.*, 2003). This suggests that AKAP149 associates with PP1 in a cell-cycle dependent manner, and has an important role in regulating PP1 function upon exit from mitosis.

AKAP149 and AKAP121 are identical in the COOH-terminal region that contains the KH and Tudor domain. The hnRNP K homology (KH) domain was first identified in the heterogenous nuclear ribonucleoprotein K (hnRNP K) 14 years ago(Siomi *et al.*, 1993). KH domain-containing proteins are responsible for specific binding/regulation of mRNAs and ssDNA in the cell, such as mRNA transport, post-transcriptional regulation of mRNA and RNA splicing (Siomi *et al.*, 1994; Nagai, 1996; Grishin, 2001; Tadesse *et al.*, 2008; Herr *et al.*, 2008; Bouvrette *et al.*, 2008). The KH domain of AKAP149/121 has also been implicated in binding to mRNA (Ranganathan *et al.*, 2002; Ginsberg *et al.*, 2003; Ranganathan *et al.*, 2005; Dyson *et al.*, 2008). The KH domain of AKAP149 binds *in vitro* to the 3' untranslated region (UTR) of several mRNAs such as the mitochondrial protein manganese superoxide dismutase (*MnSOD*), the mitochondrial ATP synthase subunit (*Fo-F)*, the steroidogenic acute regulatory protein (*mStar)* and the adipose specific lipoprotein lipase (*LPL*) (Ranganathan *et al.*, 2002; Ginsberg *et al.*, 2003; Ranganathan *et al.*, 2005; Dyson *et al.*, 2008). RNA binding is suggested to be dependent

on PKA-mediated phosphorylation of the KH domain, and AKAP121/149-anchored PKA may be responsible for this phosphorylation (Ginsberg *et al.*, 2003). The regulation exerted by AKAP149/121 on the mRNA could be individual, for instance AKAP121-*MnSOD* mRNA binding might result in higher level of *MnSOD* mRNA translocation from the cytosol to mitochondria (Ginsberg *et al.*, 2003), whereas the AKAP121-PKA-*LPL* mRNA complex leads to translational decrease of LPL protein in pre-adipocytes (Ranganathan *et al.*, 2005). The latter may explain the decrease in LPL activity following catecholamine signalling, which is known to induce PKA (Morita *et al.*, 1998; Ranganathan *et al.*, 2005; Unal *et al.*, 2008). The function of the KH domain of AKAP149, which interestingly contains a PP1-binding motif and RNA binding features in its core, is a significant part of the results presented in this thesis (Papers II, III, IV).

AIMS OF THE STUDY

Many cellular functions are regulated by reversible protein phosphorylation. cAMP-dependent protein kinase A (PKA) and protein phosphatase 1 (PP1) are two well characterized effectors involved in many essential phosphorylation-dependent processes. Accurate signaling by PKA and PP1 i.e., phosphorylation/dephosphorylation of target substrates, require precise spatio-temporal regulation of these signaling molecules. This thesis focuses on two phospho-regulating proteins, PNUTS and AKAP149. Aims of the work presented in this thesis were to:

1. Investigate the role of the PNUTS:PP1 holoenzyme on chromosome decondensation in a cell-free system.
2. Investigate the role of the KH and TUDOR domains of AKAP149 on AKAP dimerization and association with RNA, *in vivo*.
3. Further elucidate the PP1-binding features of AKAP149 in the context of its two PP1-binding motifs and of RNA association.
4. Develop a simple, rapid and efficient RNA immunoprecipitation protocol to identify novel protein-RNA interactions.

SUMMARY OF RESULTS

PUBLICATION I

PNUTS enhances *in vitro* chromosome decondensation in a PP1-dependent manner

PP1 is a Ser/Thr phosphatase involved in mitosis exit and chromosome decondensation. In this study, we characterize the subcellular and subnuclear distribution of PNUTS, a nuclear regulatory subunit of PP1. In interphase, PNUTS co-fractionates with PP1 and with chromatin. Immunofluorescence analysis indicates that PNUTS is targeted to the reforming nuclei at the end of mitosis, after nuclear envelope assembly, as chromatin decondenses. In interphase cytosolic extract, ATP-dependent decondensation of prometaphase chromosomes is blocked by PP1-specific inhibitors. In contrast, a recombinant PNUTS (309-691) fragment accelerates chromosome decondensation. Decondensation-promoting activity requires the consensus RVXF PP1-binding motif of PNUTS, whereas a secondary, inhibitory PP1-binding site is dispensable. In a buffer system depleted of cytosolic factors, PNUTS (309-691) and PP1, in the presence of an ATP generating system, promote chromosome decondensation. These results indicate an involvement of the PP1:PNUTS holoenzyme in chromatin decondensation and argues that PNUTS functions as a PP1-targeting subunit in this process.

PUBLICATION II

The KH-Tudor domain of A-kinase anchoring protein 149 mediates RNA-dependent self-association

A-kinase anchoring proteins (AKAPs) control the subcellular localization and temporal specificity of protein phosphorylation mediated by cAMP-dependent protein kinase.

AKAP149 (AKAP1) is localized in mitochondria and in the endoplasmic reticulum-nuclear envelope network, and anchors protein kinases, phosphatases, and a phosphodiesterase. AKAP149 harbors in its COOH-terminal part one K-homology (KH) domain and one Tudor domain, both known to be involved in RNA binding. In this article we show that AKAP149 self-associates in an RNA dependent manner through the KH domain. This KH domain is sufficient for self-association of AKAP149 in a RNA-dependent manner. However, the Tudor domain is required together with the KH domain for targeting to well-defined nuclear foci. These foci are spatially closely related to nucleolar subcompartments coilin and UBF. We also show that a fragment of AKAP149 containing the KH-Tudor domains of AKAP149 binds RNA *in vitro* and in RNA immunoprecipitation experiments. AKAP149 emerges as a scaffolding protein involved in intracellular signaling and possibly RNA metabolism or post-transcriptional control.

PUBLICATION III

Mutually exclusive binding of PP1 and RNA to AKAP149 affects the mitochondrial network

The K-homology (KH) domain of A-kinase anchoring protein (AKAP)149 contains an uncharacterized conserved PP1 binding RVXF motif in the RNA binding groove. In this paper we set out to define the functional importance of PP1 and RNA binding to the KH domain of AKAP149. We show that PP1 binding occurs through the conserved RVXF motif in the KH domain, and that PP1 and RNA binding to this same site is mutually exclusive and controlled through a novel, phosphorylation-dependent mechanism. A collapse of the mitochondrial network is observed upon overexpression of RNA-binding

deficient mutants in the KH domain of AKAP149. The results point to the importance of RNA tethering of the mitochondrial membrane by AKAP149 for mitochondrial distribution.

PUBLICATION IV

QuickRIP – Cross-linked RNA immunoprecipitation

Nucleic acid binding proteins constitute nearly one-fourth of all functionally annotated human genes. Genome-wide analysis of protein-nucleic acid contacts has not yet been performed for most of these proteins, restricting attempts to establish a comprehensive understanding of protein function. In this paper we aimed to identify the RNA(s) binding to the K-homology (KH) domain of A-kinase anchoring protein (AKAP)149 *in vivo*, by developing an improved cross-linked RNA immunoprecipitation (RIP) assay (QuickRIP). Our protocol is faster, easier, cleaner and applicable to a broader range of proteins than previously developed RIP protocols. RIP conditions allow us to proceed with RNA for cloning or potentially hybridization to a full-genome cDNA chip. The RIP assay reveals a phosphorylation-dependent regulation of RNA binding to AKAP149, and demonstrates direct RNA binding to AKAP149 through the conserved GxxGxxV hydrophobic pocket in the KH domain. The method provides a powerful tool to elucidate the regulation of protein:RNA binding regulation *in vivo*, and advances the probability of revealing novel protein:RNA interactions.

DISCUSSION

Phosphorylation constitutes an essential switch in many processes in the body such as cell-growth and division, neuronal function and fertility. Studies presented in this thesis aim to give insights on the function of the conserved protein phosphatase 1 (PP1) through the study of two of its regulatory subunits: PNUTS and AKAP149. Regulation of PP1- and RNA binding (Paper III), methods used (Paper I and IV) and potential biological relevance (Paper I, II, III and IV) are discussed.

A role for PNUTS in mitosis

PNUTS is involved in chromosome decondensation *in vitro*

In vitro systems to study chromosome decondensation

In vitro systems such as that used in Paper I for investigating functions of the PNUTS:PP1 holoenzyme have been used to study chromatin-related events occurring in mitosis (Lohka and Masui, 1984b; Wood *et al.*, 1990; Collas *et al.*, 1999a; Steen *et al.*, 2000a). Under proper conditions and using sperm as chromatin templates, frog egg extracts can recapitulate chromosome decondensation, NE assembly, initiation of DNA synthesis and subsequent chromosome re-condensation (Lohka *et al.*, 1984a; Lohka *et al.*, 1984b; Lohka *et al.*, 1985). Similarly, mitotic mammalian cell extracts can allow chromosome condensation from heterogenous nuclei to take place (Wood *et al.*, 1990; Collas *et al.*, 1999b; Steen *et al.*, 2000a). In Paper I, we introduce a related method where mitotic chromosome clusters isolated from nocodazole-treated HeLa cells were incubated in interphase HeLa cell cytosolic extract to study chromosome decondensation.

An advantage of such cell-free systems is that they provide experimental control by the addition and/or removal of specific components to investigate a specific function. For instance in our *in vitro* system, an ATP-generating system was necessary to enhance the efficiency of chromosome decondensation, reflecting the energy-dependent nature of this process *in vivo* (Cameron and Poccia, 1994). Alternatively, the number of unknown factors in a cell extract can be reduced by substituting the extract with a buffer containing an ATP generating system, an approach used here to identify a more direct effect of the PNUTS:PP1 holoenzyme on chromosome decondensation.

Although an *in vitro* system may provide initial indications of a biological function, it may not always reflect *in vivo* biological activities. For example, immunodepletion of condensin I from frog egg extracts inhibits *in vitro* chromosome condensation (Hirano *et al.*, 1997); however, siRNA knockdowns of condensins I and II do not inhibit completely chromosome condensation *in vivo*, indicating that condensins alone are not essential for chromosome condensation (Ono *et al.*, 2003). Nevertheless, sister chromatid separation and chromosome segregation defects were noted, arguing for important roles for condensins in the regulation of mitotic chromosome condensation (Ono *et al.*, 2003). We show here that PP1 and PNUTS together promote mitotic chromosome decondensation from chromosome clusters, both in an interphase HeLa extract and in a buffer system (Paper I). One potential bias in here could be that proteins essential for chromosome decondensation are not fully stripped from chromosome clusters during purification. Nonetheless, there is a significantly higher level of chromosome decondensation in the PNUTS:PP1 condition compared to the control conditions with PP1 alone or with other known PP1 inhibitors.

Substrate(s) of the PNUTS:PP1 holoenzyme important in chromosome decondensation remain(s) unidentified. However, several substrates of PP1 during mitosis exit, such as H1, H2A, H3, H4, Retinoblastoma protein (pRb), condensins and topoisomerase II can be considered as candidates. Since PNUTS has been reported to inhibit PP1 activity towards pRb, and hyperphosphorylation of pRb is necessary for the cells to mitosis exit (Krucher *et al.*, 2006; De *et al.*, 2008), pRb represents a very promising candidate substrate for the PNUTS:PP1 holoenzyme. However, PNUTS seems to act as an inhibitor of PP1 toward Rb dephosphorylation (De *et al.*, 2008), and in our assays the inhibitory domain of PNUTS was dispensable for chromosome decondensation. We also show chromosome decondensation in a buffer system which is devoid of pRb since cell fractionation studies have shown that pRb is soluble in mitosis and likely not associated with chromosome clusters (Nelson and Ludlow, 1997). This indicates additional role(s) for the PNUTS:PP1 holoenzyme other than to inhibit pRb in mitosis exit.

H1 and topoisomerase II have both been shown to have reduced phosphorylation levels in anaphase (Paulson *et al.*, 1996; Ishida *et al.*, 2001). Topoisomerase II is phosphorylated on four Ser residues upon mitosis entry (Hackbarth *et al.*, 2008) and could therefore potentially be dephosphorylated by PP1 at mitosis exit. Also, H1 dephosphorylation towards mitosis exit is inhibited by the PP1 and 2 inhibitor okadaic acid, suggesting PP1 as a likely candidate for H1 dephosphorylation at anaphase. Since PNUTS is a regulatory subunit for PP1 activity in mitosis exit *in vitro,* the PNUTS:PP1 holoenzyme may be involved in dephosphorylation of the potential PP1 substrates

mentioned above. However, *in vivo* substrates for the PNUTS:PP1 holoenzyme in chromosome decondensation remain to be determined.

Is PNUTS involved in checkpoint regulation in G_2?

Interestingly, after Paper I was published, the functions of the PNUTS:PP1 holoenzyme have been further examined in our laboratory in an *in vivo* context (Landsverk et al., unpublished data). Surprisingly, knockdown of PNUTS by siRNA did not delay chromosome decondensation in telophase, cells were instead delayed in G_2/prophase and showed difficulties to enter mitosis (**Fig. 10**).

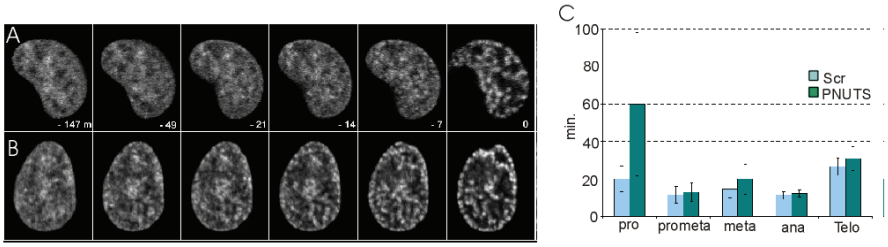

Figure 10. PNUTS depleted cells are arrested in prophase. (A and B) Scrambled siRNA transfected (A) or PNUTS siRNA (B) transfected HeLa cells were imaged by live cell confocal microscopy during entry into mitosis. Prophase is defined as the period between first visible signs of mitotic chromosome condensation and 'collapse' of chromosomes at the time of NE breakdown. Transition shown in -7 to 0 min in control condition (A) while PNUTS siRNA transfected cells remains longer in prophase (B). (C) Statistics on average time spent in the various stages of mitosis; prophase, promethaphase, methaphase, anaphase amd telophase, as indicated (Landsverk et al., unpublished data).

Furthermore, γ-irradiation of HeLa cells to activate the G_2 checkpoint revealed an increase in the population of cells delayed in G_2 for PNUTS siRNA-depleted cells (Landsverk et. al. unpublished data). It is tempting to speculate that this G_2 delay results from the impossibility for PNUTS:PP1 to dephosphorylate G_2 check-point complexes like Cdc25 or ATM, an event required for re-entry into the cell cycle and into mitosis

(Kaufmann, 1998). Also, and as mentioned earlier, PNUTS can inhibit PP1 activity towards pRb, possibly also linking PNUTS to regulation of the cell cycle by preventing mitosis exit and allowing apoptosis (De *et al.*, 2008). Collectively, recent investigations strengthen the case for an important role for PNUTS in chromatin-related regulatory events in the nucleus in mitosis, although the precise mechanisms remain unrevealed to date.

Regulation of PP1 binding and activity by phosphorylation-dependent switches in the regulatory subunits

Paper III describes a mutually exclusive phosphorylation-dependent binding of PP1 or RNA to the KH domain of AKAP149. PP1 binds when Ser630 is dephosphorylated, whereas RNA binds when Ser630 is phosphorylated. Phosphorylation events in or close to the RVXF motif of PP1 R subunits can abolish PP1 binding, or PP1 activity towards a given substrate (Margolis *et al.*, 2003; Kwiek *et al.*, 2006). The PP1-binding site in the KH domain of AKAP149 contains a conserved PKA phosphorylation site at Ser630 which potentially adds another layer to the regulation of PP1 and RNA binding. Phosphorylation of Ser287 of Cdc25, another PP1 R subunit, results in its binding to protein 14-3-3 which in turn inhibits PP1 phosphatase activity towards this residue, leading to G_2 arrest (Margolis *et al.*, 2003). Notably, Ser287 of Cdc25 can also be phosphorylated by PKA *in vitro* (Duckworth *et al.*, 2002). Interestingly, PNUTS as well harbors a conserved potential PKA phosphorylation site (Ser398) in its RVXF motif and the RVXF motif overlaps with a potential 14-3-3 binding site (**Fig. 11**). This pleads for further PNUTS:PP1 binding regulation experiments, including PP1 and 14-3-3 mutants to

study cell-cycle regulation, nucleocytoplasmic localization and cell viability (**Fig. 12**). Our findings raise the attractive possibility of a phosphorylation-dependent switch for binding of PP1 (or other proteins) or RNA in a mutually exclusive manner to the same residues on a multivalent anchoring protein. How frequent this mechanism is uncertain, but in any event, it appears to provide another level of regulation to keep PP1 activity under strict control in the cell (**Fig. 12**).

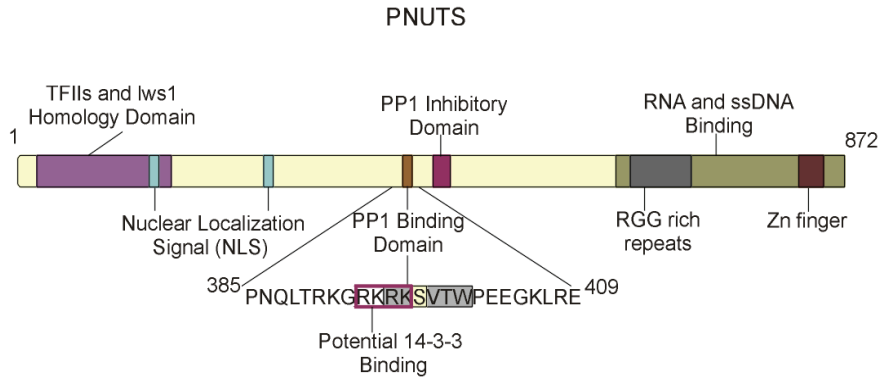

Figure 11. PNUTS with potential and known binding domains. The PP1 binding RVXF is highlighted together with a potential PP1 binding regulatory phosphorylation site. A potential 14-3-3 binding site is also shown.

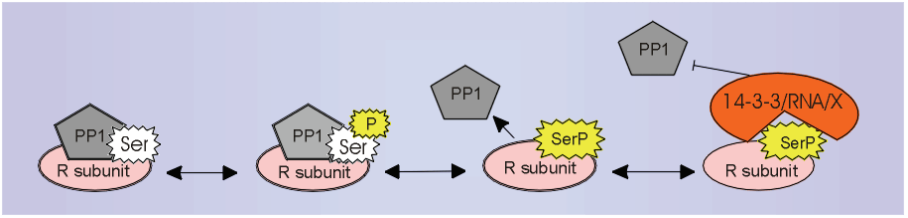

Figure 12. Hypothetical model of PP1 binding regulation to the regulatory subunit (R). When R is in a dephosphorylated state PP1 may bind. Upon phosphorylation PP1 is released from its regulatory subunit and replaced with binding to 14-3-3, RNA or an unknown protein X. Binding of 14-3-3/RNA/X to the R subunit prevents PP1 activity by inhibiting association with the PP1 R subunit or access to the phosphorylation site or both.

Presence of several PP1 binding motifs in the same regulatory subunit

In Paper III we characterize a RVXF PP1 binding motif inside the KH domain of AKAP149, and compare it to the previously described RVXF motif situated in the NH_2-terminal of AKAP149 (Steen *et al.*, 2000b). It has been shown that at least two other regulatory subunits of PP1 also have two RVXF motifs, namely Sipp1 (Llorian *et al.*, 2004) and Aurora A kinase (Katayama *et al.*, 2001). For Sipp1, both RVXF motifs seem to bind PP1 as only a double RAXA mutation of both RVXF motifs completely abolishes PP1 binding (Llorian *et al.*, 2004). Our results indicate that under our conditions the COOH-terminal RVXF motif binds PP1 significantly more that the NH_2-terminal one for which no binding was detected by immunoprecipitation. This could be due to 1) the NH_2-terminal binding site not binding PP1 under our experimental conditions, 2) the NH_2-terminal binding site not having strong enough affinity for PP1c for other binding partners already present to be displaced, 3) the NH_2-terminal associating exclusively with the δ isoform of PP1 since we only used PP1γ and PP1α in our studies, 4) the NH_2-terminal binding site binding PP1 only in very specialized cells or cellular processes, 5) wrong NH_2-terminal anchoring signal to allow PP1 binding to the NH_2-terminal 6) requirement for additional areas outside our construct to enhance PP1c binding for it to become detectable. The COOH-terminal RVXF is identical in mouse and humans, and highly conserved with only one amino-acid difference between fugu fish and humans. The NH_2-terminal RVXF is not conserved between mouse and humans, maybe suggesting a specific involvement in higher primate processes such as memory or learning. One intriguing question about the presence of two PP1-binding RVXF motifs in

the same regulatory subunit is whether they can be involved in different biological processes?

It is becoming increasingly clear that PP1 regulatory subunits contain other interaction points for PP1 in addition to the conserved RVXF motif (Bollen, 2001; Wakula *et al.*, 2003). Moreover, the presence of the RVXF motif alone is not enough to affect the activity or conformation of PP1 in a noticeable way; therefore additional binding points are necessary for a specific regulatory-subunit effect on PP1. It has been proposed that multiple binding domains to PP1 in the regulatory subunits restrict the accessibility of the catalytic site by using steric hindrance or by inducing conformational changes (Bollen, 2001; Wakula *et al.*, 2003). These changes are able to affect the activity and substrate specificity of PP1. In Paper I, we observe that the PP1-binding, but not the PP1 inhibitory, domain of PNUTS is required to allow chromosome decondensation *in vitr*o. This implies that PNUTS could have another role than to inhibit PP1 in the process of chromosome decondensation *in vitro*, possibly by acting as a substrate specifier for PP1 and enhancing PP1 activity towards one or several PP1 substrates in chromatin related processes in mitosis. If several PP1 binding regions exist in PNUTS, PNUTS could respond differently to cell signaling events by association with PP1 either in a substrate specifier, targeting subunit or inhibitor of PP1 context. The exact nature of PNUTS:PP1 binding and regulation remains to be revealed.

The KH domain as a RNA-binding and a PP1-binding domain

We indicate in Paper III that binding of PP1 and RNA to the KH domain occurs through overlapping residues, is mutually exclusive and inversely controlled by phosphorylation. This is to our knowledge the first time RNA and PP1 bind in a mutually exclusive way to one and same domain. Until now, it had only been reported that PP1 binds directly to a RVXF motif located in the RNA recognition motif (RRM) of tra2-beta1 (Novoyatleva *et al.*, 2008), but PP1 binding was not shown to depend on RNA binding and no evidence for binding regulation by phosphorylation was provided. Eight other proteins including SF2/ASF and SRp30c contain an evolutionary conserved PP1 RVXF motif in their RRM, indicating that PP1 binding is a frequent feature of some RNA binding domains (Novoyatleva *et al.*, 2008). The discovery of PP1 binding to RNA binding motifs opens for a more profound and broader role for PP1 in RNA metabolism, splicing and regulation than previously suggested.

AKAP149 self associates through the KH domain in a RNA-dependent manner, suggesting a role for AKAP149 in post translational control

RNA dependent self-association

KH domains bind RNA or ssDNA, and are found in proteins associated with transcriptional and translational regulation, splicing and other cellular processes (Siomi *et al.*, 1994; Grishin, 2001; Tadesse *et al.*, 2008). The typical binding surface of a KH domain consists of a cleft formed by four unpaired amino acids (GXXG) where van der Waals forces, hydrophobic- and electrostatic interactions contribute to nucleic acid binding affinity (Valverde *et al.*, 2008). Augmented KH domain or multiple KH domain

copies are two strategies used to achieve greater affinity and specificity of nucleic acid binding in a protein. In Paper II, we show that AKAP149 binds RNA *in vivo* and self-associates in a RNA-dependent manner through the KH domain, as RNAse digestion of the extract before immunoprecipitation greatly reduces self-association. RNA-dependent self-association has also been indicated for *X. laevis* Vg1RBP (homologue to human KOC protein used as a marker for malignancy in cancer), another KH domain protein (Git and Standart, 2002). We show in Paper III that mutations in the KH domain RNA-binding cleft (V629E or V629AF631E) almost abolishes RNA binding, and the V629E mutation also reduces self-association (Paper II). One question remaining is how the V629E mutant with strongly reduced RNA binding only shows ≈50% reduction in self-association? There are several possible explanations 1) *in vivo* cross-linking experiments as in Paper III reflect RNA bound to AKAP149-EGFP in intact 293 cells, while V629E mutation studies in Paper II performed in HeLa cell extracts could result in a larger RNA pool of RNA molecules available to associate with AKAP149 2) the V629E mutation may result in lower affinity to RNA, possibly too weak or sterically unfavourable for UV cross-linking, but still associated to RNA to some extent. One way to explore these possibilities further would be to perform *in vivo* cross-linking followed by RIP on wild-type or RNA-binding deficient mutants of the KH domain and visualize the protein RNA complexes by SDS-PAGE (essentially as performed in Paper III). *In vivo* cross-linking before immunoprecipitation would allow us to look for dimer or trimer complexes of AKAP149-RNA and observe how these are affected by mutations in the RNA binding groove of the KH domain (V629E or F629AF631A). Collectively, our results indicate

that AKAP149 self-associates in a RNA dependent manner through the GxxG binding groove of the KH domain.

RNA binding to AKAP149 – preference for methylated residues?

An interesting feature of KH domains is that they always are more similar to KH domains with comparable positions in other proteins than to other KH domains from the same protein (Grishin, 2001). Also, KH domains within the same protein have different affinity towards the selection of RNAs binding to the protein (Git *et al.*, 2002). AKAP149 only contains one KH domain but as indicated in paper II stabilizes RNA binding by self-association through the KH domain and possibly also through the Tudor domain. Tudor is a domain involved in RNA/DNA and protein interactions with a preference for methylated residues (Huyen *et al.*, 2004; Cote and Richard, 2005). RNA binding to KH often occurs in a dimer or tetramer of different KH domains with distinct affinities for a given RNA. Thus a hypothesis is that RNA binding to two identical KH domains provided by the AKAP149 dimer would increase the strength of binding to RNA. The Tudor domain would provide additional selectivity. Interestingly, AKAP149 contains, between the KH and Tudor domain, a conserved potential WW domain binding motif (^{686}PPLP^{689}) known to preferentially bind methylated residues (Ryan and Bauer, 2008). Moreover, WW domains are implicated in binding to a subset of splicing factors including the KH domain-containing hnRNP H, hnRNP M and hnRNP E1, and are able to associate to the 3' part of an intron to form a complex equivalent to the early spliceosome (Lin *et al.*, 2004). Whether AKAP149 associates with a WW domain-containing protein remains unknown at this stage, but one possibility to elucidate this

would be to perform initial binding studies with recombinant KH-Tudor fragments of AKAP149 on a WW domain array membrane. In the TranSignal™ WW Domain array (Panomics, cat no. MA3030) protein interactions take place directly on the WW domain array membrane, where potential interaction can be visualized using chemiluminescence. The presence of a conserved KH domain, Tudor domain and a potential WW domain binding motif, could suggest the COOH-terminal part of AKAP149 is involved in selective binding to RNA for post-transcriptional control or for transport to its proper localization. The KH, Tudor and WW domain binding motifs also open for the possibility of a fine tuned RNA-binding regulation to AKAP149 depending on the methylation mark of the RNA, with methylation of certain residues enhancing RNA binding to AKAP149, and with phosphorylation of the KH domain a pre-requisite for RNA binding (Paper III).

Possible new unexplored nucleic acid binding features of AKAP149

In this thesis we explored RNA- and PP1-binding through the COOH-terminal but leave another intriguing possible feature of AKAP149 - DNA binding - unexplored. It is an attractive hypothesis to test whether AKAP149 binds chromatin, as both the KH domain and the Tudor domain have DNA binding properties in other proteins. For instance, DDP1 homologue to the highly conserved Vigilin protein, contains 15 KH domains and associates preferentially to centromeric satellite DNA sequences in *D. melanogaster*, and further, mutations in the protein suppress heterochromatin mediated silencing (Cortes and Azorin, 2000; Steen *et al.*, 2000b; Birchler *et al.*, 2004). The Tudor domain has also been demonstrated in several instances to bind DNA, in the context of DNA damage response and transcriptional regulation of genes by binding to modified histones. For example, the

tandem Tudor domains of 53BP1 are recruited to double-stranded DNA breaks and bind to methylated histone H3-K79 (Huyen *et al.*, 2004). Furthermore, a double Tudor domain of Jmic domain-containing histone demethylase (JMJD2A) binds methylated histone H2-K4 and H4-K20 (Huang *et al.*, 2006). Notably, when examining the AKAP149 sequence there are several other domains potentially implicated in DNA binding in addition to the KH and Tudor domain. This includes the above mentioned WW binding domain shown to be involved in preferential binding to methylated DNA(Ingham *et al.*, 2005; Brucet *et al.*, 2007). In addition, AKAP149 contains 12 residues corresponding to the 12 first residues of a chromodomain (amino-acid 260-271); a domain widely known to have important roles in DNA binding and gene regulation (Nielsen *et al.*, 2004; Jacobs *et al.*, 2004; Lomberk *et al.*, 2006). But the small size of this fragment makes it questionable whether it is functional. AKAP149 also contains a leucine-zipper (amino-acid 304-340), implicated in chromatin binding, gene regulation, DNA damage response and apoptosis (Wang *et al.*, 2006; Miotto and Struhl, 2006; Ciccone *et al.*, 2008). Presence of all the motifs mentioned above strongly suggest a possible role for AKAP149 in DNA binding and possibly gene regulation. However, one issue to resolve might be to reveal first AKAP149 localizations in the cell more precisely, especially how entry into the nucleus is possible and how AKAP149 is retained there. Taken together, the possible DNA binding features of AKAP149 remain unexplored to date, but open for new important biological functions for the AKAP149 scaffolding protein.

Towards a model: AKAP149 - mRNA transport - posttranscriptional control

The RNA-dependent self-association of AKAP149 suggests a role for AKAP149 in RNA transport and or post-transcriptional regulation. One attractive scenario may be that mRNA molecules sandwiched between two AKAP149 molecules are protected and transported to their proper subcellular localization, for instance mitochondria (**Fig 13**). Transport of mRNA from the nucleus to mitochondria by AKAP149 has also been suggested for the two mitochondria-specific mRNAs *MnSOD* and *FoF* (Ginsberg *et al.*, 2003). Binding of mRNA to AKAP149 has further been suggested to occur through the 3' UTR of several mRNAs (Ranganathan *et al.*, 2002; Ginsberg *et al.*, 2003; Ranganathan *et al.*, 2005; Dyson *et al.*, 2008). More generally, it is also increasingly clear that mRNA localization is a mechanism involved in the sorting of proteins destined to organelles, especially in mitochondrial biogenesis (Sylvestre *et al.*, 2003). In *S. cerevisiae*, half of the transcripts coding for mitochondrial proteins preferentially localize to the organelle surface (Sylvestre *et al.*, 2003), and this is also observed in human cells (Corral-Debrinski, 2007). There are also reports showing poly-ribosomes transported to a specific subcellular localization to translate proteins in close proximity to their final localization such as cytoskeleton compartments and neuronal dendrites (Schratt *et al.*, 2004). The RNA species localized to the mitochondria through AKAP149 binding could be released from AKAP149 upon a signal promoting AKAP149 Ser630 dephosphorylation by a Ser/Thr phosphatase, possibly PP1, making AKAP149 unable to associate with RNA (Paper III). Since AKAP149 does not contain a nuclear localization signal, the question remains to know how then can AKAP149 enter the nucleus to bind RNA? One answer could be that AKAP149 binds a transport molecule which allows

AKAP149 to enter the nucleus. It is for instance known that binding to a WW domain protein can alter cell localization, as is the case for human NIMA-related kinase 6 (Nek6). Nek6 usually localize to RNA speckles in the nucleus but mutations in the WW binding domain of PPLP result in Nek6 no longer co-localizing to speckles (Lee *et al.*, 2007a). Also, AKAP149 contains a potential leucine-zipper which can promote nuclear import in the absence of a nuclear import signal by binding to other leucine-zipper containing proteins (Waldmann *et al.*, 2007). Inside the nucleus, AKAP149 probably remains bound to molecules that keep it in the nucleus while binding to RNA, and upon release from these factors the seven potential nuclear export signals present in the AKAP149 sequence will cause exit from the nucleus. Binding to RNA requires AKAP149 to be phosphorylated on Ser630 (Paper III), therefore AKAP149 may be phosphorylated by PKA or another kinase on Ser630 in the nucleus, before binding to its specific RNA. When AKAP149 re-enters the cytoplasm, RNA can again be transported to its proper localization, here mitochondria, where binding is retained until the RNA release signal reappears (**Fig. 13)**.

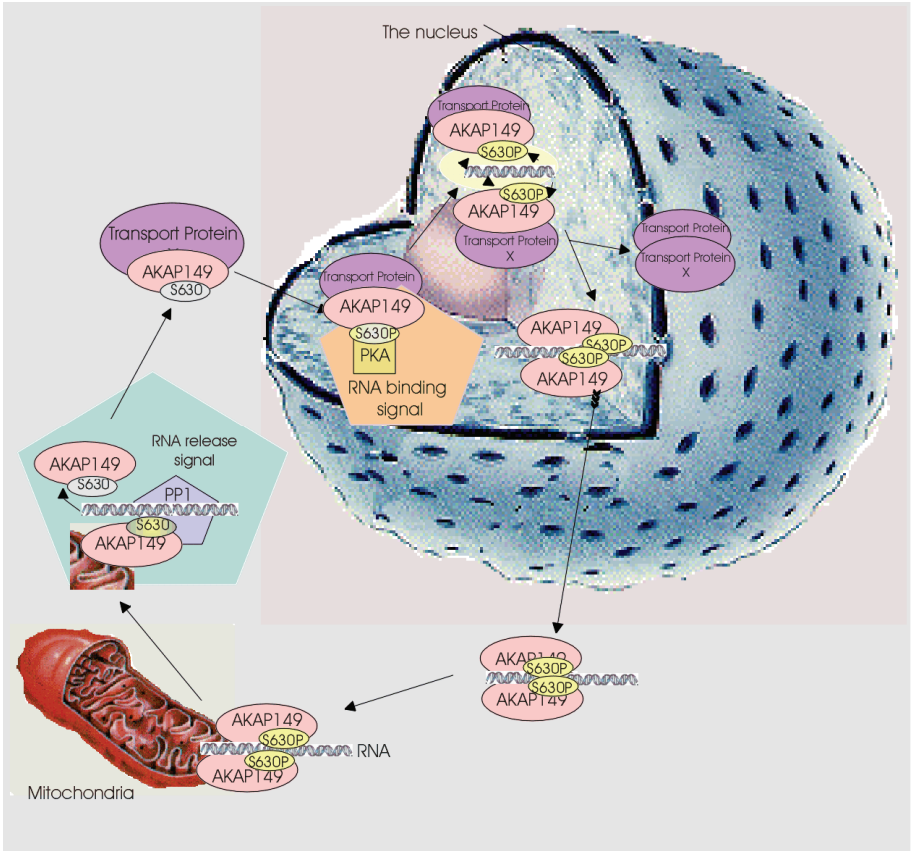

Figure 13. Hypothetical model for RNA binding to AKAP149 in the cell. RNA is sandwiched between two AKAP149 molecules in the mitochondria, and upon a signal possibly leading to KH domain dephosphorylation by PP1, RNA is released from AKAP149 and can be translated. AKAP149 binds a transport protein X (X = unknown) which allows AKAP149 entry into the nucleus. In the nucleus AKAP149 rebinds RNA after Ser630 phosphorylation of AKAP149, possibly by PKA. Following release from the transport protein X, AKAP149 exits the nucleus (AKAP149 contains at least seven potential nuclear export signals). The AKAP149-RNA complex is then transported to its proper localization depending on AKAP149 NH_2-terminal splice-variant and bound RNAs 3'UTR localization sequence, which in our example localizes to the mitochondria. (Modified from mac122.icu.ac.jp/gen-ed/eukaryotic-cell.html)

RNA-binding to the KH domain of AKAP149 - QuickRIP

To determine the nature of RNAs binding AKAP149 *in vivo*, we optimized an RNA immunoprecipitation assay (QuickRIP) for AKAP149, described in Paper IV. Immunoprecipitation of AKAP149 was performed after UV-cross-linking and partial T_1RNAse digestion of the RNA, to obtain RNA fragments with proper length for linker ligation, cloning and sequencing. Of note, 85% of the RNAs in a cell are ribosomal, thus creating a substantial risk of false positive hits in screening attempts. Even though we improved RIP to reduce false positive rRNA hits by the use of magnetic beads, and two linker ligations on the beads, it remains difficult to distinguish the real binding to rRNAs from false positives.

RIP of AKAP149 shows strong enrichment of RNA binding specifically and directly to AKAP149. Surprisingly, the identified RNA-binding hits for AKAP149 *in vivo* were solely ribosomal RNAs (**Table. 1**). None of the mRNAs previously shown to bind AKAP149 *in vitro* were found (Ranganathan *et al.*, 2002; Ginsberg *et al.*, 2003; Ranganathan *et al.*, 2005; Dyson *et al.*, 2008). Furthermore, one of the ribosomal (5.8S) RNA hits was also found once in the control (**Table. 1**). Taken together, the rRNA hits are interesting, but it remains to be shown whether obtained rRNA are false positives or real binding partners of AKAP149.

rRNA	Control	RIP
18S	0	1
5.8S	1	10
28S	0	8

Table 1. RNA obtained for AKAP149 after UV-cross-linking, immunoprecipitation, cloning and identification of the bound eluted RNA by sequencing. Results are identifiable RNA' obtained from five independent RIP experiments.

What would be the implications of AKAP149 really binding ribosomal RNA? It has been known for a long time that 5.8S RNA needs to be methylated for protection before transport to the mitochondria (Munholland and Nazar, 1987; Abou and Nazar, 1997). Furthermore, a correlation between level of 5.8S RNA methylation and cellular fate has been shown where by reduced ribosomal 5.8S RNA methylation correlates with differentiation and development of cancer (Abou *et al.*, 1997). The cell cycle regulator and tumor suppressor p53 binds covalently to 5.8S and causes ribosomal stress, aborted protein synthesis and p53-dependent cell-cycle arrest (Munholland *et al.*, 1987; Abou *et al.*, 1997; Pestov *et al.*, 2001). Dysregulation of p53-induced ribosomal stress is correlated with several types of cancers (Gilkes and Chen, 2007). Based on our screen we may hypothesize that the KH domain of AKAP149 is involved in protection and transport of 5.8S RNA from the nucleus to the mitochondria. Since AKAP149 also contains a Tudor domain known to preferentially bind methylated residues, the Tudor domain would restrict binding to 5.8S RNA with the proper methylation pattern. To our knowledge, it has not been shown that 18S and 28S have methylated RNAs, but it is known that the upstream binding factor (UBF) dependent transcription of all ribosomal genes in the nucleus by topoisomerase II is significantly affected by the level of ribosomal gene methylation (Preuss and Pikaard, 2007). Interestingly we observe in paper II that the KH-Tudor fragment of AKAP149 localizes in close proximity to UBF in the nucleus of HeLa cells, and that UBF co-immunoprecipitates both with the KH-Tudor fragment of AKAP149 and with endogenous AKAP149. Taken together, our QuickRIP results may suggest a role for AKAP149 in the expression- or post-transcriptional regulation of ribosomal RNA in the cell.

One way to solve remaining issues that regards rRNA hits would be to amplify immunoprecipitated nucleic acids for hybridization to genome-scale cDNA chips. RIP hits should also be supported by other methods like *in vitro* RNA assays, immunofluorescent RNA experiments or PCR with hit-specific primers to be confirmed. By analogy to the widely used chromatin immunoprecipitation (ChIP) assay, RIP assays can potentially be highly informative provided one resolves the issue of unspecific background or false-positive sequencing hits.

RNA deficient mutants of AKAP149 display mitochondrial collapse

In Paper III we performed mutation analysis to elucidate the features of the KH domain of AKAP149, and observed that RNA-binding deficient mutants of AKAP149, when transiently-overexpressed in HeLa cells, result in a collapsed mitochondrial phenotype. The 1-485 fragment of AKAP149 lacking the KH domain shows the strongest phenotype with the mitochondrial network gathered in a compact accumulation on one or both sides of the nucleus. Moreover, mitochondria appear as small dots rather than with the elongated widely dispersed aspect they present in normal cells. This fragmentation suggests a possible involvement for AKAP149 in the mitochondrial fission and fusion processes.

The mitochondrial network is in a constant equilibrium between mitochondrial fission and fusion, where a constant mitochondrial fusion regulated by the mitofusins proteins is necessary to prevent the mitochondrial population in the cell from becoming highly fragmented (Chen and Chan, 2004). Mitofusins are conserved, large GTPases localized to the mitochondrial outer membrane (Santel and Fuller, 2001; Rojo *et al.*,

2002; Chen *et al.*, 2003). Mammals have two closely related mitofusin homologues, Mfn1 and Mfn2. Cells lacking Mfn1 or Mfn2 have greatly reduced levels of mitochondrial fusion (Chen *et al.*, 2003; Chen *et al.*, 2005). In our mutant expression assays, mitochondrial collapse phenotype is observed as early as 8 hours after transfection. This corresponds to a previous report that shows mitochondrial fusion covering the entire mitochondrial network in a human cell within 12 hours, and also that blocking of mitofusins or mitofusin-related proteins display mitochondrial fragmentation after 4 hours (Legros *et al.*, 2002). Overexpression of Mfn1 or/and Mfn2 has been shown to be able to rescue cells with a fragmented mitochondrial phenotype (Santel *et al.*, 2001; Chen *et al.*, 2003). Thus, further investigations to elucidate a possible rescue of the collapsed mitochondrial phenotype by expression of Mfn1 and/or Mfn2 would be required to link this phenotype to a known biological process.

Further use of mutants showed that it is the lack of RNA binding to AKAP149 which leads to a collapsed mitochondrial network. Requirement of correct RNA localization for normal cell morphology and viability is a relatively unexplored field. However another KH domain-containing protein, Moep19, is suggested to direct RNAs essential for normal embryo-development to specific locations in the oocyte and early embryo (Herr *et al.*, 2008). Also, mutations in the KH domain of the fragile X mental retardation product (FMR1) are correlating with the development of fragile X syndrome, especially in the more severe cases. Fragmented or collapsed mitochondrial network has often been correlated with loss of mitochondrial membrane potential and apoptosis (Chen *et al.*, 2005; Huang *et al.*, 2007) and one intriguing question would be whether loss of RNA binding to AKAP149 affects viability?

Although mitochondrial network collapse or fragmentation usually affects viability of the cells (Chen *et al.*, 2003;Huang *et al.*, 2007), this is not always the case. During the late stages of sperm maturation, a massive mitochondrial compaction is shown to be very important for sperm maturation and viability (Cardullo and Baltz, 1991). In addition, a deletion mutation in the GTPase domain of autosomal dominant optic atrophy (OPA1), causes an mitochondrial network collapse and fragmentation, but does not seem to increase the level of apoptosis (Spinazzi *et al.*, 2008). Mitochondrial network abnormalities caused by OPA1 have been suggested as a cause for autosomal dominant optic atrophy – the most common cause of inherited optic atrophy. Interestingly, AKAP84, a testis specific, shorter splice variant of the AKAP1(149) gene and lacking the KH domain, is kept under strict developmental control in testis. AKAP84 expression levels increase rapidly during the last steps of sperm maturation as the mitochondria gather into the midpiece (Reinton *et al.*, 2000). Strikingly, AKAP149 is hardly expressed in testis at all. All together, this leads to the postulate that AKAP84 has a role in spermatogenesis that requires the absence of the KH domain. A role for AKAP84 in spermatogenesis is conserved from fugu fish to humans (Reinton *et al.*, 2000), and as previously mentioned so is the RNA binding groove (but not the PP1 binding RVXF motif), further supporting the idea of the lack of RNA binding to the AKAP84 being required for normal testis maturation. In this context it would be interesting to over-express full-length AKAP149 in sperm and look for effects on sperm maturation. Another question arises from the fact that it is important to keep AKAP84 under strict control and just expressed in sperm as the mitochondria wanders towards the midpiece. Could this open for disease-related studies based upon disrupted mitochondrial

network by RNA deficient mutants of AKAP149, perhaps without affecting cell viability? This could concern diseases linked to tissues where mitochondria are specifically important like brain or muscle (Filosto *et al.*, 2007).

PERSPECTIVES

It is estimated that 1 person per 8,000 has a disease caused by induced mutations affecting mitochondrial function and processes, and mitochondrial dysfunctions are currently being implicated in a growing amount of diseases (Tarnopolsky and Raha, 2005). For instance errors in the mitochondrial fatty acid oxidation causes diseases characterized by distinct enzyme or transport deficiencies, leading to liver dysfunction and cardiomyopathy, among others (Kompare and Rizzo, 2008). Also, lipid metabolism disorders, Parkinsons disease, inherited diabetes mellitus and myopathy are all linked to mitochondrial dysfunction (Schocke *et al.*, 2008; Kompare *et al.*, 2008; Gal *et al.*, 2008; Mitsui *et al.*, 2008; Bruno and Dimauro, 2008; Vanitallie, 2008). Mitochondria also play essential roles in natural aging, viral infection and metabolism – all areas where AKAP149 potentially could play important roles. The impact AKAP149 exerts on mitochondrial functions and cell viability remains to be demonstrated, and can potentially link AKAP149 to one or more mitochondrial dysfunction caused disease in the future.

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